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         Coactivator AIB1 in Breast Cancer

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13. ABSTRACT (Maximum 200 Words)

In this proposal we have investigated the hypothesis that overexpression of a novel truncated version of AIB1 (Δ3AIB1) that we have found to be overexpressed in breast cancer is import for tumor development by impacting upon nuclear hormone receptor function. We have examined the hypothesis that the novel AIB1 variant has an altered function that changes its interaction with nuclear receptors such as the estrogen or progesterone receptor. We propose that changes in the level of expression of the novel AIB1 variant will support tumor progression and may well have prognostic significance for breast cancer. We have now determined that Δ3AIB1 is overexpressed relative to the full-length protein in breast cancer tumor samples and cell lines. We have determined that Δ3AIB1 is a significantly more active coactivator than full-length AIB1 on the estrogen and progesterone receptor (*J. Biol. Chem.* 276, 39736-39741, 2001). We have shown that Δ3AIB1 overexpression increases estrogenic activity in agonists and SERMs such as tamoxifen (*Oncogene*, in press 2003) In addition, a surprising finding is that overexpression of Δ3AIB1 can also potentiate EGF signaling. This implies that Δ3AIB1 can also drive non-hormone mediated proliferation in breast cancer.

14. SUBJECT TERMS

AIB1, estrogen receptor, breast cancer, Δ3AIB1

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Table of Contents

Introduction

An area of chromosome 20q, frequently amplified in breast cancer harbors a steroid receptor coactivator (SRC) gene, AIB1 (amplified in breast cancer 1). (1) SRCs are a family of proteins that bind to the liganded receptor and when overexpressed are able to increase the maximal response to steroids such as estrogen and progesterone. (2) The AIB1 gene is amplified in 5-10% of breast cancers and the mRNA is overexpressed in about 60% of tumors. (1) We have also determined that the AIB1 protein is overexpressed in breast cancer by immunocytochemical analysis. (3) The role of AIB1 is further complicated by overexpression studies that demonstrate that AIB1 cannot only potentiate the action of the estrogen and progesterone receptor but also a number of other nuclear receptors in transient transfection over expression assays. (2) Interestingly, some of these receptors such as retinoid and vitamin D_3 have predominantly anti-proliferative and pro-apoptotic roles in breast cancer whereas other receptors such as estrogen, progesterone and thyroid receptor are considered to induce growth by increased proliferation and reduced apoptosis. Thus it is possible to envisage the overexpression of AIB1 as enhancing both proliferative and antiproliferative effects of nuclear receptors. It is also apparent that the effects of the SRC coactivators are not confined to interactions with the nuclear receptor family. The SRC proteins also interact with other cofactors such as the pCAF andCBP/p300 cointegrators (4) that are recipients of signals from a number of cellular signaling pathways. In addition, AIB1 has been described as interacting with p53. (5) Thus, it is entirely possible that AIB1 may have wide ranging effects on cell cycle and proliferation that are independent of its effects on nuclear receptor function.

Another aspect of nuclear coactivators which is particular interesting is the existence of exon splice variants of the SRC-1 and SRC-3 (AIB1) genes that can be predicted from the cDNA cloning data, although the levels of expression and impact of the presence of these alternative splice products on coactivator function is not known (2). For ACTR/AIB1, such putative splice variants can be predicted within the receptor interacting region and in the N-terminal helix-loop-helix-(HLH)-Per-Arnt-Sim (PAS) domain. We have now detected a novel Δ exon 3 AIB1 splice variant that is expressed in MCF-7 breast cancer cells as well as in breast tumor tissue. (appendix manuscript) We have determined the exon intron structure of AIB1 from the human genome database and the predicted protein product from this isoform is an N-terminally truncated version of AIB1 that has a deleted HLH domain and most of the PAS region removed. The predicted size of this protein is approximately 125 kDA and a protein with this molecular weight is detected in our Western blot analysis of MCF-7 cells and is translated *in vitro* from the Δ exon 3 AIB1 cDNA.

To our knowledge this is the first report of expression of an AIB1 splice variant at the mRNA and protein levels in breast cancer cells. Alterations in the domain are of interest since it has been shown that the PAS/HLH domain can mediate homodimerization between family member and in this case may mediate crosstalk between nuclear receptors and PAS/HLH family members that have wide ranging effects in cellular growth control.

In this grant we have examined the hypothesis that the Δ exon 3 splice AIB1 variant has an altered function, due to the removal of the PAS/HLH domain, which changes its interaction with nuclear receptors or other transcription regulators. We propose that changes in the level of

expression of Δ exon 3 AIB1 will support tumor progression and will likely have prognostic significance for breast cancer.

Body

Statement of Work

Task 1. Analyze the function of the Δ exon 3 AIB1 isoform versus full-length AIB1 in transient transfection assays.

We first compare the Δ exon 3 AIB1 to the full length AIB1 protein with respect to their ability to potentiate nuclear receptor function in transient transfection assays in transformed and non-transformed breast cell lines.

We have now completed a series of experiments showing the Δ exon 3 isoform is a significantly more effective transcriptional coactivator of both the estrogen receptor and the progesterone receptor. We have recently had a paper accepted (*Oncogene*, in press) showing that Δ3AIB1 potentiates partial agonists (tamoxifen) and natural estrogens (genistein). See appendix manuscript. In addition, we have now shown that the Δ exon 3 AIB1 variant potentiates epidermal growth factor signaling. The data we have obtained on the functional impact of the expression of Δ exon 3 AIB1 in transient systems and the expression data showing that Δ exon 3 mRNA is highly overexpressed in both breast cancer cell lines and breast tumor samples has now been published in a *J Biol Chem* paper and this manuscript can be found in the appendix of this report.

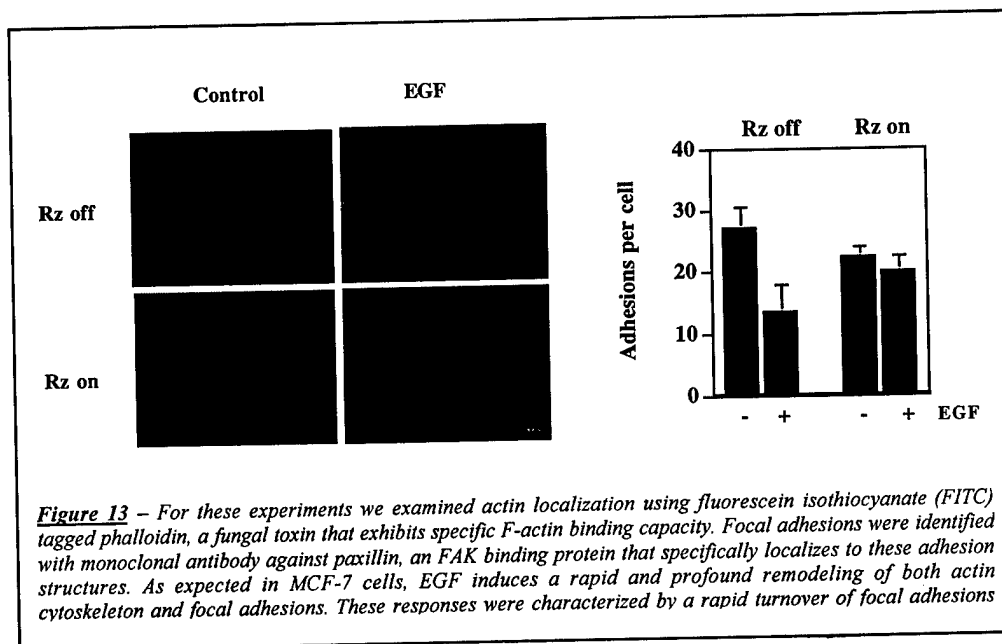### Task 2. Analyze the effect of stable expression of Δ exon 3 AIB1.

We will examine the impact of the expression of Δ exon 3 AIB1 on normal breast epithelial and on breast cancer cell phenotype by developing cell lines stably expressing the Δ exon 3 AIB1 or full-length AIB1. We will focus in particular on malignant transformation in the normal epithelial cells and on basal and hormone-induced proliferation and apoptosis in the cancer cells.

This task is currently in progress. We are trying to create cell lines that stably expressing the Δ exon 3 and to help us with that we have now made a tagged version of the Δ exon 3 expression vector both with a histidine tag at the C-terminus and also a MYC tag at the C- terminus to determine if we have cell lines that clearly overexpress Δ exon 3. We have tried to make cell lines overexpressing Δ3AIB1 and these cells do not survive. We just succeeded in making - Δ3AIB1 Tet inducible stable cell lines and now will examine changes in these levels on phenotype and tumorigenicity.

Task 3. Determine the effect of overexpression of Δ exon 3 AIB1 on angiogenic and invasive properties of breast cancer cells.

We will use cell lines developed under *Task 2* to determine the effects of Δ exon 3 AIB1 on stromal and endothelial cell interactions with breast tumor cells.

We have some preliminary data showing that reducing AIB1 in cells does impact on the invasive behavior of tumors (Fig. 1) and will now extend this with the Tet-inducible cell lines.

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Figure 13 – *For these experiments we examined actin localization using fluorescein isothiocyanate (FITC) tagged phalloidin, a fungal toxin that exhibits specific F-actin binding capacity. Focal adhesions were identified with monoclonal antibody against paxillin, an FAK binding protein that specifically localizes to these adhesion structures. As expected in MCF-7 cells, EGF induces a rapid and profound remodeling of both actin cytoskeleton and focal adhesions. These responses were characterized by a rapid turnover of focal adhesions*

For this we are encouraged that this Δ exon 3 AIB1 splice variant will have a major impact on the angiogenic and invasive phenotype on MCF-7 cells and will continue to persevere with this task during the next year, using the cell lines we are currently developing under *Task 2*.

Key Research Accomplishments

Task 1: Completed in MCF-7 cells. *J. Biol. Chem.* article attached. *Oncogene* manuscript attached.

Task 2: Underway

Task 3: Will be started as soon as cell lines available.

Reportable Outcomes

Article

Reiter, R., Oh, A.S., Wellstein, A. and **Riegel, A.T.** Impact of the Nuclear Receptor Coactivator AIB1 Isoform AIB1-Δ3 on Estrogenic Ligands with Different Intrinsic Activity. Accepted, *Oncogene,* 2003.

Reiter, R., Wellstein, A., and **Riegel, A.T.** "An Isoform of the Coactivator AIB1 that Increases Hormone and Growth Factor Sensitivity is Overexpressed in Breast Cancer." (2001) *J. Biol. Chem.* 276, 39736-39741.

An Isoform of the Coactivator AIB1 That Increases Hormone and Growth Factor Sensitivity Is Overexpressed in Breast Cancer*

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The AIB1 (amplified in breast cancer 1) protein is a coactivator that potentiates the transcriptional activity of nuclear hormone receptors, and its gene is amplified in a subset of human breast cancers. Here we report a splice variant of *AIB1* mRNA that lacks the exon 3 sequence. We determined that the *AIB-Δ3* mRNA encoded a 130-kDa protein that lacks the NH_2-terminal basic helix-loop-helix and a portion of the PAS (Per-Arnt-Sim homology) dimerization domain. The 130-kDa protein was detected in MCF-7 breast cancer cells at levels that were 5–10% of the full-length protein, whereas in non-transformed mammary epithelium lines, the AIB-Δ3 protein was present at significantly lower levels compared with the full-length AIB1. Consistent with this finding, the abundance of *AIB1-Δ3* mRNA was increased in human breast cancer specimens relative to that in normal breast tissue. To determine whether there were phenotypic changes associated with the overexpression of the AIB-Δ3 isoform, we performed functional reporter gene assays. These revealed that the ability of AIB1-Δ3 to promote transcription mediated by the estrogen or progesterone receptors was significantly greater than that of the full-length protein. Surprisingly, the AIB1-Δ3 isoform was also more effective than AIB1 in promoting transcription induced by epidermal growth factor. Overexpression of AIB1-Δ3 may thus play an important role in sensitizing breast tumor cells to hormone or growth factor stimulation.

Ligands such as estrogen and progesterone that interact with nuclear receptors regulate gene expression predominantly at the transcriptional level. The ligand-bound receptors interact specifically with DNA and activate transcription by recruiting a preinitiation complex. Although such gene activation was originally thought to be mediated by interaction of the receptors with components of the basal transcriptional machinery (1–6), a variety of screening techniques has identified a family of receptor-interacting proteins known as nuclear receptor coactivators (7–11). A common characteristic of this superfamily of proteins is that, when overexpressed in the presence of nuclear receptors, they potentiate ligand induction of transcription (12, 13). Members of the related p160 group of coactivators, which include steroid receptor coactivator 1 (SRC-1),[1]

SRC-2, and SRC-3 (also known as AIB1, ACTR, RAC3, TRAM-1, and p/CIP) (14–20), possess several similar structural features including a receptor interaction domain, a bHLH (basic helix-loop-helix)-PAS (Per-Arnt-Sim homology) dimerization domain, and a CBP interaction domain (13). Coactivators are thought to function as bridges between nuclear receptors and either other coactivators or the basal transcriptional machinery (13). However, the discovery that coactivators possess a histone acetylase domain (15, 21–24) suggests that these proteins also might serve to regulate chromatin structure.

A portion of human chromosome 20q that is frequently amplified in breast cancer contains the gene for the nuclear coactivator AIB1 (amplified in breast cancer 1) (25). The *AIB1* gene is amplified in 5–10% of breast cancers, and the abundance of the corresponding mRNA and protein is increased in some breast tumors and breast cancer cell lines (14, 25–27). It has recently been shown that AIB1 binds directly to the estrogen receptor (ER) (28) and that AIB1 is rate-limiting for estrogen-induced growth of MCF-7 cells (29). However, the overall role of AIB1 for breast tumorigenesis is not clear because AIB1 potentiates not only the action of estrogen (14, 16) and progesterone (16) receptors but also that of various other nuclear receptors (9, 15, 17–20) and transcription factors (30, 31). In addition, several splice variants of SRC family members have been described, although the functions of these variants remain unknown (13).

Here we report the identification of a splice variant of *AIB1* that is overexpressed in breast cancer tissue and cell lines. The *AIB-Δ3* transcript encodes an NH_2-terminal truncated version of AIB1 that lacks the bHLH and PAS A domains. In functional studies we have determined that the AIB-Δ3 protein is a significantly more effective coactivator of estrogen, progesterone, and EGF signaling than the wild type ER, suggesting a role for this AIB1 isoform in hormone and paracrine signaling in breast cancer.

EXPERIMENTAL PROCEDURES

Plasmids—We subcloned the full-length *AIB1* cDNA from pCMX-ACTR (provided by R. Evans, Salk Institute, La Jolla, CA) into pcDNA3 (Invitrogen) with the use of the flanking *Kpn*I and *Xho*I sites, thereby creating the expression vector pcDNA3-AIB1. We subcloned the smaller of the two RT-PCR products generated from MCF-7 cell total RNA with exon 1- and exon 9-specific primers (Fig. 1*b*) into pCRII (Invitrogen). The resulting plasmid was digested with *Bam*HI and *Hpa*I, recognition sequences that flank the splice sites of *AIB1-Δ3* cDNA, and the released fragment was purified and used to replace the corresponding sequence of pcDNA3-AIB1, thereby creating pcDNA3-AIB1-Δ3. The pcDNA3-

[1] The abbreviations used are: SRC, steroid receptor coactivator; bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim homology; ER, estrogen receptor; ERE, estrogen response element; PCR, polymerase chain reaction; RT, reverse transcription; IMEM, Iscove's modified Eagle's medium; FBS, fetal bovine serum; EGF, epidermal growth factor; CHO, Chinese hamster ovary; FGF-BP, fibroblast growth factor-binding protein; TEF, transcription-enhancing factor.

AIB1 and pcDNA3-AIB1-Δ3 vectors contain identical 5′- and 3′-untranslated regions, differing only in the loss of exon 3 in the latter. The inserts were verified by sequencing.

The expression vectors for human estrogen receptor α and progesterone receptor B were provided by P. Chambon (CNRS, France). The firefly luciferase reporter plasmid containing the estrogen response element (ERE) from the *Xenopus* vitellogenin gene was provided by V. C. Jordan (Northwestern University, Chicago), and the plasmid containing the mouse mammary tumor virus promoter was provided by G. Hager (National Cancer Institute, Bethesda, MD). The luciferase reporter plasmid containing the human *FGF-BP* gene promoter has been described previously (32). The *Renilla* luciferase vector (pRL-CMV) was from Promega.

Cells and Tissue Samples—All cell lines were obtained from the tissue culture core facility of the Lombardi Cancer Center. MCF-7, ME-180, and COS-1 cells were cultured in Iscove's modified Eagle's medium (IMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS). MCF-10A and A1N4 cells were grown in a 1:1 mixture of IMEM and Ham's F-12 medium (Life Technologies, Inc.) that was supplemented with 5% horse serum, EGF (20 ng/ml), insulin (10 μg/ml), and hydrocortisone (500 ng/ml). CHO cells were maintained in F-12 nutrient mixture (Life Technologies, Inc.) supplemented with 10% FBS.

Frozen tissue samples were obtained from the Lombardi Cancer Center Histopathology and Tissue Shared Resource Core. The six normal samples were obtained from individuals undergoing reduction mammoplasty (mean age at time of surgery, 29 years; range, 19 to 54 years); the eight primary breast carcinoma specimens were obtained from women with a mean age at the time of surgery of 51 years (range, 29 to 64 years).

Immunoblot Analysis—Whole cell extracts were prepared as described previously (32), and equal portions (30 μg of protein) were resolved either on denaturing 4–20% polyacrylamide gradient gels or on 4% polyacrylamide gels containing Tris-glycine. The separated proteins were transferred to a nitrocellulose membrane and then subjected to immunoblot analysis with a 1:500 dilution of a mouse monoclonal antibody specific for amino acids 376–389 of human AIB1 (Transduction Laboratories), horseradish peroxidase-conjugated goat antibodies to mouse immunoglobulin (1:10,000 dilution; Amersham Pharmacia Biotech), and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

RT-PCR—Isolation of total RNA and synthesis of cDNA by RT were performed as described previously (33). The amplification of *AIB1* cDNA sequences was achieved by PCR according to the following protocol: incubation at 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 90 s. The oligonucleotides used as primers for PCR or as probes for hybridization were as follows: exon 1, 5′-GACTGGTTAGCCAGTTGCTG-3′; exon 2, 5′-GCCATGTGATACTCCAGGAC-3′; exon 3, 5′-CTGAGCTGATATCTGCCAATC-3′; exon 4, 5′-AGCCGATGTATCTTCTACAGG-3′; exon 5, 5′-ATGTTTCCGTCTCGATTCACC-3′; exon 8, 5′-CCTCATGGAGGATCTCAGTG-3′; and exon 9, 5′-CCATCAGCCAACGAGAATCG-3′. The PCR products were separated by electrophoresis on a 1% agarose gel, transferred to a polyvinylidene difluoride membrane, and hybridized with a 32P-labeled oligonucleotide probe. Quantification of PCR products was performed with a PhosphorImager (Molecular Dynamics 445SI).

Transient Transfection and Reporter Gene Assays—COS-1 and CHO cells were plated at densities of 2 × 10⁵ and 5 × 10⁵ cells/well, respectively, in six-well plates and were cultured for 24 h at 37 °C under 5% CO₂ in IMEM or Ham's F-12, respectively, supplemented with 5% FBS that had been treated with dextran-coated charcoal. The medium was then replaced with IMEM containing LipofectAMINE Plus (Life Technologies, Inc.) and expression and reporter plasmids as indicated. After incubation for 3 h, the medium was replaced with IMEM (COS-1 cells) or Ham's F-12 (CHO cells), each containing 5% dextran-coated charcoal-treated FBS and nuclear receptor ligands. Cells were incubated for 24 h and then disrupted in passive lysis buffer (Promega). Portions (20 μl) of the resulting cell extract were assayed for both firefly and *Renilla* luciferase activities with the Dual-Luciferase reporter assay system (Promega).

ME-180 cells were plated at a density of 5 × 10⁵ cells/well and cultured for 24 h in IMEM supplemented with 5% dextran-charcoal-treated FBS. They were then incubated for 3 h in IMEM supplemented with LipofectAMINE Plus and expression and reporter plasmids. The cells were washed and then incubated in IMEM for an additional 3 h before incubation for 18 h with EGF (5 ng/ml) in serum-free IMEM and subsequent lysis. Because of high background induction of pRL-CMV expression by EGF, firefly luciferase activity was normalized by protein
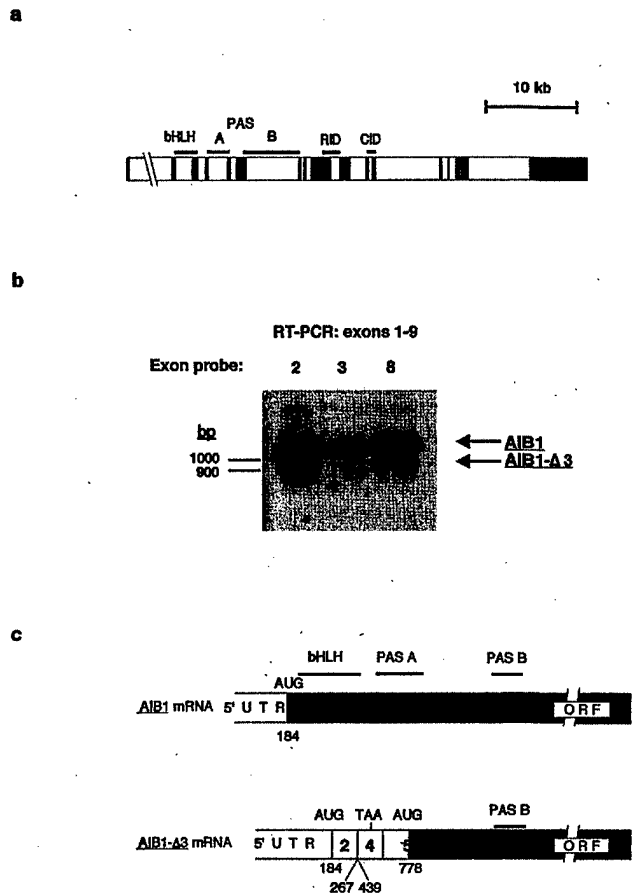
FIG. 1. Characterization of a splice variant of human *AIB1*. *a*, structure of human *AIB1* showing the 22 known exons (*filled boxes*) and the corresponding introns (*open boxes*). The exon/intron regions, which are spliced to form the various functional domains of the AIB1 protein, are indicated by *horizontal bars*. *b*, detection of the *AIB1-Δ3* splice variant in total RNA from MCF-7 cells. The total RNA was subjected to RT-PCR with primers specific for exons 1 and 9 of *AIB1*. The reaction products were resolved on a 1% agarose gel and transferred to a polyvinylidene difluoride membrane, which was then cut, and the lanes were subjected separately to hybridization with 32P-labeled oligonucleotides specific for exons 2, 3, or 8 of *AIB1*. The positions of PCR products corresponding to the full-length (*AIB1*) and truncated (*AIB1-Δ3*) transcripts are indicated. *bp*, base pairs. *c*, comparison of the structures of *AIB1* and *AIB1-Δ3* mRNAs. The alternative splicing event that results in the loss of exon 3 causes the open reading frame (*ORF*) to shift and terminate at a TAA codon in exon 4. A potential initiation site (*AUG*) for *AIB1-Δ3* mRNA is present at nucleotide 778; the use of this site would be consistent with the AIB-Δ3 protein lacking the NH₂-terminal 26 kDa of full-length AIB1. The *shaded* regions indicate the open reading frame, and exons in the mRNAs are *numbered*. The positions of the splice junctions in *AIB1-Δ3* mRNA and of the encoded protein domains are indicated. *UTR*, untranslated region.

concentration as described previously (32).

In Vitro Transcription-Translation—In vitro transcription-translation was performed with the TnT coupled reticulocyte lysate system (Promega). Plasmid DNA (1 μg) was combined with 25 μl of rabbit reticulocyte lysate, 2 μl of TnT reaction buffer, 1 μl of T7 RNA polymerase, 1 μl of amino acid mixture, 1 μl of Rnasin (Ambion) ribonuclease inhibitor (40 units), and 1 μl of Transcend biotin-lysyl-tRNA (Roche) and the final volume was adjusted to 50 μl. The reaction was performed at 30 °C for 90 min, after which 5 μl of the reaction mixture was subjected to SDS-polyacrylamide gel electrophoresis and either to immunoblot analysis with antibodies to AIB1 or to direct detection with streptavidin-conjugated horseradish peroxidase (1:10,000 dilution in phosphate-buffered saline containing 0.05% Tween 20) and enhanced chemiluminescence.

RESULTS

Detection of the AIB1-Δ3 Isoform—In this study we determined whether there are naturally occurring splice variants of
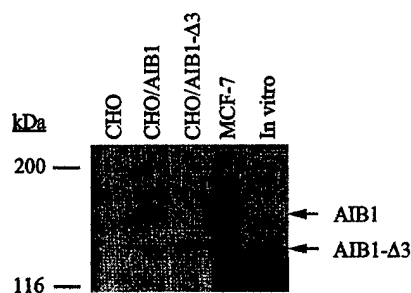
FIG. 2. **Immunoblot analysis of AIB1 isoforms in extracts of MCF-7 cells and transfected CHO cells.** Extracts of untransfected CHO cells or CHO cells transfected with plasmids encoding AIB1 or AIB1-Δ3, or MCF-7 cells were fractionated by electrophoresis on high resolution 4% polyacrylamide gels containing Tris-glycine, and the separated proteins were transferred to a nitrocellulose membrane and probed with a monoclonal antibody specific for amino acids 376–389 of human AIB1. The products of *in vitro* transcription-translation of *AIB1-Δ3* cDNA were similarly analyzed.

AIB1 present in breast cancer cells that might encode proteins with altered function relevant to breast cancer progression. The exon-intron structure of *AIB1* was assembled as shown in Fig. 1a by comparing the published sequence of the cDNA (14) with the contiguous genomic sequence available through the NCBI data base. We arbitrarily designated the most 5' exon of *AIB1* as exon 1, with the result that the first codon is located in exon 2. Our initial strategy was to determine whether RNA from MCF-7 cells, which overexpress AIB1 (14), contained any splice variant forms of AIB1 RNA. To achieve this end, we performed reverse transcription and polymerase chain reaction (RT-PCR) analysis of total RNA from MCF-7 human breast cancer cells with primers amplifying the region between exons 1 and 9. This revealed two PCR products that differed in size by ~150 base pairs. These PCR products were then subjected to Southern blot analysis, and individual lanes from the membrane were probed separately with oligonucleotides specific for each exon from 2 to 8. Typical hybridizations with exons 2, 3, and 8 are shown in Fig. 1b. This analysis revealed that the smaller PCR product hybridized with all probes except the one specific for exon 3 (Fig. 1b), indicating that the lower band corresponds to an RNA splice variant (designated *AIB1-Δ3*) of *AIB1* that lacks the exon 3 sequence. We subsequently subcloned and sequenced this PCR product, confirming that nucleotides 267–439 (exon 3) of the full-length *AIB1* cDNA were missing (Fig. 1c).

Translation of the AIB1-Δ3 mRNA in Vitro and in Vivo—To determine whether an AIB1-related protein was encoded by the *AIB1-Δ3* mRNA, we performed *in vitro* transcription and translation of *AIB1-Δ3* cDNA. Western blot analysis with an AIB1-specific antibody of the proteins translated *in vitro* revealed the production of a 130-kDa protein (Fig. 2). Interestingly, we had also consistently detected a similar 130-kDa protein, in addition to the 156-kDa full-length AIB1, by immunoblot analysis of MCF-7 cell extracts with antibodies to AIB1 on 5–20% polyacrylamide gels (27). To determine whether the MCF-7 130-kDa species and the *in vitro* transcription translation product had identical electrophoretic properties, we performed high resolution electrophoresis on 4% polyacrylamide gels containing Tris-glycine followed by immunoblot analysis. This analysis demonstrated that the mobility of the 130-kDa protein detected in MCF-7 cell extracts was identical to that of the 130-kDa protein produced by *in vitro* transcription and translation of *AIB1-Δ3* cDNA (Fig. 2). This observation suggested that the 130-kDa MCF-7 cell protein was translated from *AIB1-Δ3* mRNA present in these cells.

To verify that the *AIB1-Δ3* mRNA was translated *in vivo* we performed transient transfection of CHO cells (Fig. 2; see Fig.

4a) or COS-1 cells (see Fig. 5a) with the *AIB1-Δ3* cDNA. Analysis of cell extracts demonstrated that this indeed resulted in the production of a 130-kDa protein, whereas transfection with the full-length *AIB1* cDNA yielded only the 156-kDa full-length protein. This latter observation demonstrated that the 130-kDa protein was clearly not the product of proteolytic processing of the full-length protein. The electrophoretic mobility of the 130-kDa protein synthesized in cells transfected with the *AIB1-Δ3* cDNA was identical to that of both the 130-kDa AIB1 species present in MCF-7 cell extracts and the product of *in vitro* transcription-translation of the *AIB1-Δ3* cDNA (Fig. 2). Together these data indicated that the endogenous *AIB1-Δ3* mRNA present in MCF-7 cells encodes a 130-kDa protein.

An examination of the sequence of *AIB1-Δ3* mRNA indicated that the open reading frame of *AIB1*, which initiates at nucleotide 184 in the full-length mRNA would terminate after 90 amino acids in the splice variant (Fig. 1c). We did not detect this predicted low molecular mass product *in vivo* or *in vitro*. The 130-kDa species is detected by an AIB1 antibody raised against amino acids 376–389 in the amino terminus of the protein. This suggests that the AIB1-Δ3 isoform most likely represents an NH_2-terminally truncated form of AIB1, with synthesis being initiated at an internal translation start site downstream of the splice junction but prior to amino acid 376. Such internal translational initiation has been described for various mRNAs with extended 5'-untranslated regions (34–37). The difference in size between the 156-kDa full-length AIB1 protein and the 130-kDa species suggested that the latter lacks ~210 amino acids of the former, including all of the bHLH region (residues 16–88) and most of the PAS A domain (residues 116–171) (Fig. 1c). This would place the initiation codon for the 130-kDa protein most likely at the codon at position 778 (Fig. 1c). Interestingly, for cells transfected with equivalent amounts of cDNA, the intracellular concentration of AIB1-Δ3 protein was ~10% of that of full-length AIB1 (Fig. 2; see Figs. 4a and 5a), suggesting that translation initiation of the splice variant was inefficient, possibly because of the long 5'-untranslated region of the *AIB1-Δ3* mRNA.

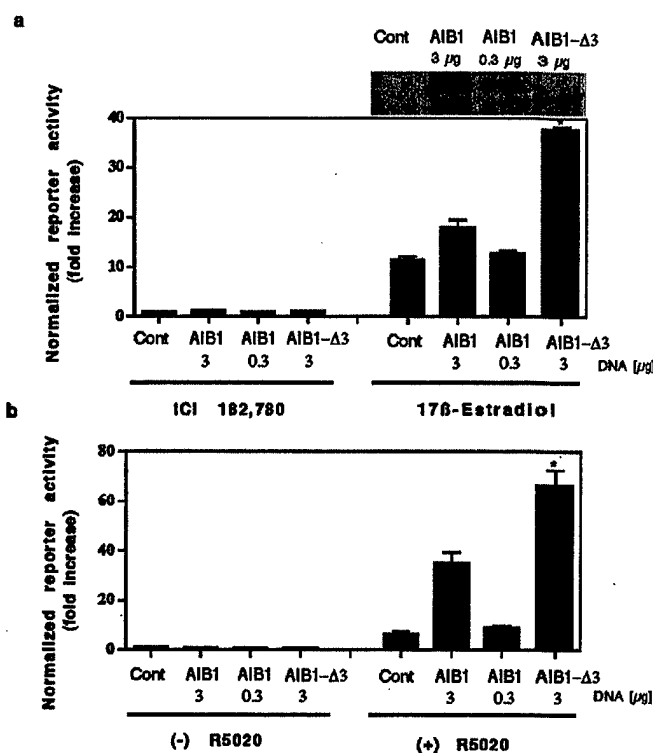*AIB1-Δ3 mRNA Is Overexpressed in Human Breast Cancer*—Given that we first detected the *AIB1-Δ3* splice variant in a breast cancer cell line, we next examined whether its expression was restricted to tumor cells. MCF-7 cells are derived from a pleural effusion of metastatic breast cancer, whereas MCF-10A and A1N4 cells are not malignantly transformed and were derived from atypical human breast epithelial hyperplasia (38) and from human mammary epithelial cells treated with benzopyrene (39), respectively. RT-PCR followed by Southern blot analysis revealed that the amounts of *AIB1-Δ3* mRNA in MCF-10A and A1N4 cells were lower than that of MCF-7 cells (Fig. 3a). By subsequent real-time PCR analysis, using primers specific for AIB1 or its isoform, we have assessed that the ratio of *AIB1-Δ3* mRNA/full-length AIB1 is 5% in MCF-7 cells, whereas in MCF-10A and AIN4 cells the ratio is 0.5% (data not shown). We then compared the abundance of the *AIB1-Δ3* mRNA in a series of eight human breast tumors with that in normal tissue obtained from six women undergoing breast reduction mammoplasty. The amount of the full-length *AIB1* mRNA in tumor samples was slightly greater than that in the normal tissue samples, but this difference was not significant (Fig. 3b). In contrast, the abundance of the *AIB1-Δ3* mRNA in the tumor specimens was significantly greater than that in the normal tissue samples, with all but one of the tumors showing an increased amount of this transcript compared with the normal range.

Effect of the AIB1-Δ3 Isoform on Nuclear Receptor Function—We next examined the effect of the deletion of the bHLH
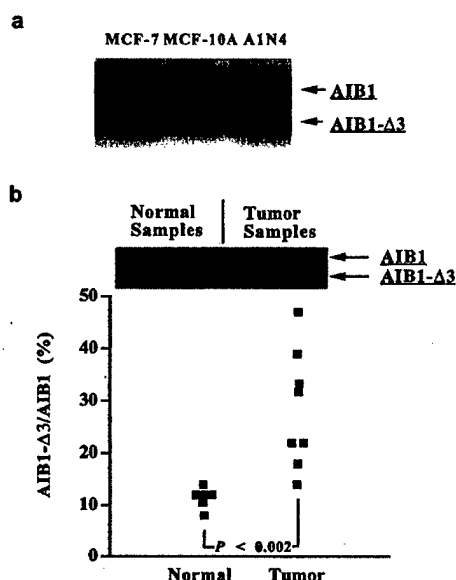
FIG. 3. **Comparison of the abundance of *AIB1-Δ3* mRNA in malignant and nonmalignant human breast tissue and cell lines.** *a*, total RNA isolated from MCF-7, MCF-10A, and A1N4 cells was subjected to RT-PCR with primers specific for exons 2 and 5 of *AIB1*. The reaction products were resolved on a 1% agarose gel and then subjected to Southern blot analysis with a ^{32}P-labeled oligonucleotide probe specific for exon 4 of *AIB1*. *b*, total RNA isolated from six normal breast and eight breast cancer tissue samples was analyzed as in *panel a*. The amounts of PCR products corresponding to *AIB1* and *AIB1-Δ3* mRNAs were quantitated by densitometry, and the abundance of the latter was expressed as a percentage of that of the former. The signal of the full-length *AIB1* transcript was compared between breast tumors and normal breast tissue with the use of an arbitrary scale; the signals in tumor and normal samples were 1.0 ± 0.46 and 0.7 ± 0.24 (means $\pm$ S.E.), respectively, and they did not differ significantly ($p > 0.05$; Student's t test). The *inset* shows a typical blot of 8 of the 14 samples.

FIG. 4. **Effects of AIB1 and AIB1-Δ3 on the activation of estrogen receptor α and progesterone receptor B in CHO cells.** *a*, cells were transfected with either the empty pcDNA3 vector (3 μg) (*Cont*), pcDNA3-AIB1 (0.3 or 3 μg), or pcDNA3-AIB1-Δ3 (3 μg) together with an expression vector for human estrogen receptor α (100 ng), an ERE-luciferase reporter plasmid (1 μg), and pRL-CMV (0.1 ng). After incubation for 24 h with either 10 nM 17β-estradiol or 100 nM estrogen receptor antagonist ICI 182,780, cells were lysed and assayed for luciferase activity. The *inset* shows immunoblot analysis of transfected cell lysates that were fractionated on 4–20% polyacrylamide gradient gels and probed with antibodies to AIB1. *b*, cells were transfected as in *panel a* with the exception that the estrogen receptor vector was replaced with a vector for human progesterone receptor B (20 ng) and the ERE-luciferase plasmid was replaced by a luciferase reporter construct containing the mouse mammary tumor virus promoter (2 μg). Cells were incubated for 24 h in the absence or presence of the progesterone analog R5020 (1 nM) before preparation of lysates for luciferase assay. The firefly luciferase activity of cell lysates was divided by the *Renilla* luciferase activity (internal control), and this ratio (normalized reporter activity) for control cells incubated in the absence of agonist was assigned a value of 1. Data are means $\pm$ S.E. of values from three independent experiments, each performed in triplicate. *, $p < 0.005$ *versus* corresponding value for cells transfected with 3 μg of the AIB1 vector (Student's t test).

and PAS A domains in AIB1-Δ3 on protein function. AIB1 acts as a coactivator for several nuclear receptors, including those for estrogen and progesterone, which are important in breast carcinogenesis. We therefore transfected CHO cells with expression vectors encoding full-length AIB1 or AIB1-Δ3, an expression vector for estrogen receptor α, and a luciferase reporter plasmid containing an ERE. Transfection of CHO cells with 3 μg of the AIB1 expression vector resulted in a 1.4-fold increase in estrogen-induced luciferase activity, whereas transfection with 3 μg of the vector for AIB1-Δ3 resulted in a 3.8-fold increase in the estrogen response (Fig. 4a). However, given that the abundance of recombinant AIB1 in the transfected cells was about 10 times that of AIB-Δ3, we also transfected CHO cells with 0.3 μg of the AIB1 vector, which yielded about the same amount of intracellular recombinant protein as did 3 μg of the AIB1-Δ3 vector (Fig. 4a). A comparison of transfected cells containing approximately equal amounts of recombinant protein thus revealed that AIB1 and AIB1-Δ3 potentiated the estrogen response by factors of 1.1 and 3.8, respectively. Similar transfection experiments with COS-1 cells (which express endogenous AIB1) also demonstrated a greater potentiation of the estrogen response by AIB1-Δ3 than by full-length AIB1 (Fig. 5a). The differences between full-length and the AIB1-Δ3 isoform were seen at different concentrations of estrogen (0.1–10 nM) and thus were not due to a change in the affinity of the hormone for its receptor but rather suggest enhanced efficacy of the signaling (data not shown). We also obtained similar results in COS-1 cells with an expression vector encoding progesterone receptor B; the transcriptional response to the progesterone analog R5020 was thus potentiated to a greater extent by AIB1-Δ3 than by AIB1 in both CHO and COS-1 cells (Figs. 4b and 5b). Of particular note is that small amounts of

transfected AIB-Δ3 protein had significant effects on ER- and progesterone receptor-induced transcription even against a relatively high background of full-length AIB1 (Fig. 5, *a* and *b*).

Effect of the AIB1-Δ3 Isoform on EGF Signaling—The fact that members of the p160 SRC family act as coactivators in intracellular signaling pathways that activate transcription factors other than nuclear receptors (30, 31) prompted us to examine whether AIB1-Δ3 might be able to sensitize breast cancer cells to growth factor signaling. Overexpression of members of the families of epidermal growth factor (EGF) ligands or EGF receptors is important in the malignant progression of breast cancer (40). Such growth factors also contribute to the hormone-independent phenotype of breast tumors and the HER-2 receptor is a target of current therapies (41). To determine whether AIB1 and AIB1-Δ3 affect EGF signaling, we transfected ME-180 human squamous cell carcinoma cells with the respective expression vectors and with a luciferase reporter plasmid containing the promoter of the fibroblast growth factor-binding protein (*FGF-BP*) gene. FGF-BP functions as an

a

ICI 182,780   17β-Estradiol

b

(-) R5020   (+) R5020
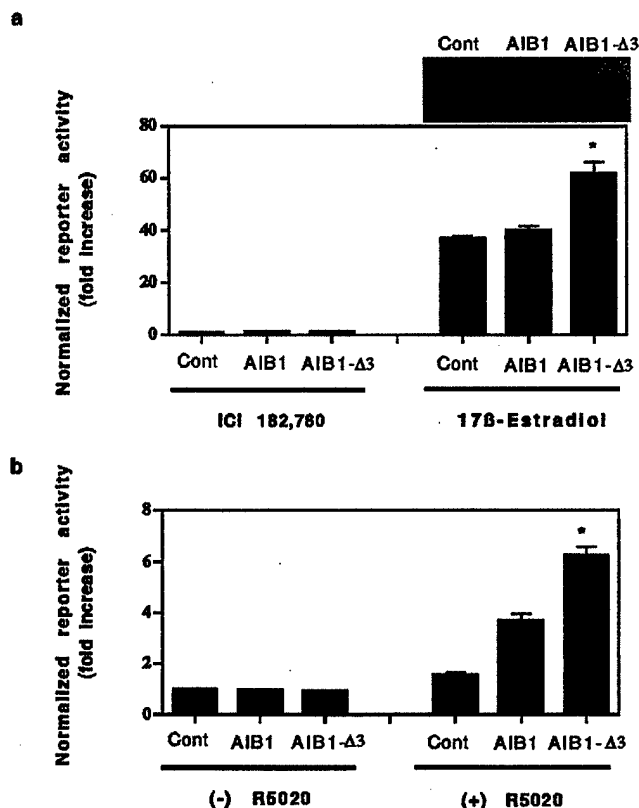
FIG. 5. **Effects of AIB1 and AIB1-Δ3 on the activation of estrogen receptor α and progesterone receptor B in COS-1 cells.** *a*, cells were transfected and analyzed as described in the legend for Fig. 4*a* (the amount of pcDNA3-AIB1 was 3 μg). *b*, cells were transfected with 1 μg of either pcDNA3, pcDNA3-AIB1, or pcDNA-AIB1-Δ3 together with an expression vector for human progesterone receptor B (10 ng), a luciferase reporter plasmid containing the mouse mammary tumor virus promoter (1 μg), and pRL-CMV (0.1 ng). After incubation for 24 h in the absence or presence of 0.5 nM R5020, cells were lysed and assayed for luciferase activity. Data are means ± S.E. of values from three independent experiments, each performed in triplicate. *, *p* < 0.005 *versus* the corresponding value for cells transfected with the AIB1 vector.

angiogenic switch molecule (42) that is overexpressed in breast cancer, and its gene is activated by EGF in squamous cell and breast cancer cell lines (32). As reported previously, EGF induced a 2.5-fold increase in reporter activity in control cells transfected with the empty expression vector (Fig. 6). The basal EGF induction was increased slightly by transfection of the full-length AIB1 expression vector, whereas EGF induction was increased ~6-fold by expressing recombinant AIB1-Δ3.

DISCUSSION

In this study we have provided evidence for the presence of a splice variant of AIB1 that has exon 3 deleted. The *AIB1-Δ3* mRNA is translated *in vivo* in breast cancer cells into an NH₂-terminal truncated form of AIB1 that has several properties of interest. The first is that on a per molecule basis it is a more potent transcriptional coactivator of both the estrogen and progesterone receptors than the full-length AIB1 protein. This result was unexpected given that previous studies of NH₂-terminal deletion mutants of the AIB1-related protein SRC-1 did not reveal an impact of this region on nuclear receptor signaling (9, 30). One possible reason for the increased activity of AIB1-Δ3 is that the conformation of this isoform is more favorable than that of the full-length protein for interaction with nuclear receptors or for recruitment of other coactivators such as CBP/p300. An alternative possibility is suggested by the observation that the bHLH-PAS domain of SRC-1 interacts
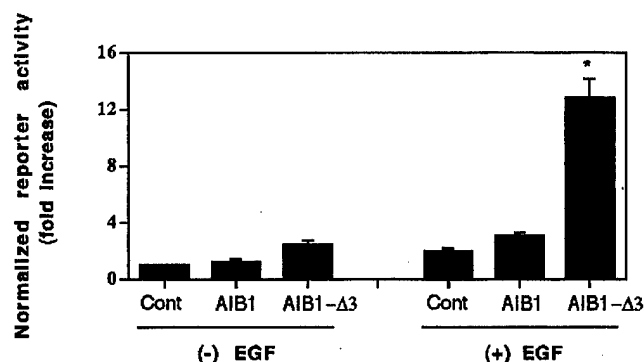
FIG. 6. **Effects of AIB1 and AIB1-Δ3 on activation of the FGF-BP gene promoter by EGF in ME-180 cells.** Cells were transfected with 3 μg of either pcDNA3, pcDNA3-AIB1, or pcDNA3-AIB1-Δ3 together with a luciferase reporter plasmid containing the human *FGF-BP* gene promoter (1 μg). After incubation of cells for 18 h in the absence or presence of EGF (5 ng/ml) in serum-free medium, cell extracts were prepared and assayed for luciferase activity. Activity was normalized by protein concentration, and the normalized activity values were then expressed relative to that of cells transfected with pcDNA3 and not exposed to EGF. Data are means ± S.E. of values from three independent experiments, each performed in triplicate. *, *p* < 0.01 *versus* the corresponding value for control cells.

with and potentiate the activity of members of the TEF (transcription-enhancing factor) family of transcription factors (30). Thus, full-length AIB1 might be unavailable for interaction with nuclear receptors because it is sequestered or squelched by other intracellular factors. In contrast, AIB1-Δ3, which lacks an intact bHLH-PAS domain, would not bind to factors such as TEF and would be available for nuclear receptor coactivation. This notion might explain why relatively small amounts of recombinant AIB1-Δ3 are able to induce significant potentiation of nuclear receptor activity in transfected COS-1 cells with a high background of endogenous full-length AIB1. This model also predicts that the relative coactivating effects of AIB1 and AIB1-Δ3 might be cell type-specific, depending on the endogenous expression of AIB1-sequestering molecules such as TEF. Interestingly, a recent report has described that the human MMS19 protein can interact with the PAS-HLH domain of AIB1 and can regulate ER-mediated transcriptional activity (43). It is possible that the lack of interaction of AIB1-Δ3 with this protein may explain some of its increased effectiveness *in vivo*. Whatever the reason for the increased activity of the AIB1-Δ3 isoform, our data suggest that its expression would sensitize cells to the effects of estrogen and progesterone.

The second interesting aspect of the function of the AIB1-Δ3 isoform was that it also potently increased EGF signaling in ME-180 squamous carcinoma cells. This could be through direct interactions with a nuclear receptor. However, our analysis of the fragment of the *FGF-BP* gene promoter (nucleotides −118 to +62, relative to the transcription start site) used in this study did not reveal obvious consensus recognition sites for known nuclear receptors. In fact, EGF induction of this promoter is dependent on the factors AP-1 and c/EBPβ (32), either of which may interact directly or indirectly with AIB1. Alternatively it may be that a common intermediary of both nuclear receptor and AP-1 signaling such as CBP/p300 (44, 45) may be the target of the superactivating effects of the AIB1-Δ3 isoform. Whatever the mechanism of the increased potentiation of growth factor signaling by the AIB1-Δ3 isoform, our data suggest that an increase in the abundance of the AIB1-Δ3 isoform in mammary epithelial cells may be an important step in tumor progression and in the development of a more aggressive, hormone-independent phenotype.

Finally, of major interest for breast cancer is our finding that the *AIB1-Δ3* mRNA is overexpressed in breast cancer cell lines

and in human breast tumors. Our analysis of tumor cell lines suggests that there is an overall increase in the *AIB1-Δ3* mRNA relative to the full-length AIB1, although we do not know whether this is related to the gene amplification status of the endogenous gene. Alternatively, the increase in *AIB1-Δ3* mRNA may be because of an increase in RNA splicing of exon 3 in breast cancer cells. It is also possible that the increase in expression in tumors may be due in part to dilution effects of surrounding stromal tissue, but this seems unlikely given that we also see lower *AIB1-Δ3* mRNA expression in non-transformed *versus* malignant mammary epithelial cell lines. To date a number of laboratories, including ours, have reported overexpression of *AIB1* mRNA and protein in breast tumor tissue, although the assessment of the portion of breast cancers overexpressing AIB1 varies widely between groups (14, 27, 46, 47). In addition, some groups have determined that AIB1 overexpression is correlated with ER and progesterone receptor status (26), whereas others have found an inverse relationship with steroid receptor expression but a positive correlation with HER-2 and p53 expression (47). However, all of these RT-PCR or immunohistochemical analyses of expression levels have not distinguished the AIB1-Δ3 isoform signal from that of the wild type. Our data indicates that the overexpression of relatively low levels of the AIB1-Δ3 isoform can sensitize cells to estrogen, progesterone, and growth factors. Therefore we believe that measurement of increased levels of AIB1-Δ3 levels might be a sensitive indicator of the progression of breast cancer to a more hormone-independent phenotype.

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Impact of the Nuclear Receptor Coactivator AIB1 isoform AIB1-Δ3 on estrogenic ligands with different intrinsic activity.

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Abstract

The nuclear receptor coactivator AIB1 (<u>a</u>mplified <u>in</u> <u>b</u>reast cancer <u>1</u>) and its more active isoform AIB1-Δ3 are overexpressed in breast cancer and preneoplastic breast tissue. However, the impact of these proteins on the transcriptional activity of natural estrogens or SERMs (selective estrogen receptor modulators) has not been determined. Here we show that AIB1-Δ3 causes a significant increase in the efficacy of 17β-estradiol at both estrogen receptor-α (ER-α) and ER-β in ovarian, breast and endometrial cancer cell lines. AIB1-Δ3 also significantly increased the efficacy of the natural estrogen genistein at both ER-α and ER-β, whereas AIB1 had no effect on either the potency or efficacy of genistein at either receptor. The estrogenic efficacy of the partial agonist tamoxifen was significantly increased in all cell lines at ER-α by overexpression of AIB1-Δ3 both on transfected and endogenous estrogen responsive genes. In contrast, overexpression of AIB1 or AIB1–Δ3 had no effect on the potency or efficacy of the SERM raloxifene. We conclude that overexpression of the AIB1–Δ3 isoform will increase the estrogenicity of a variety of natural and pharmacologic compounds in tissues that develop hormone-dependent neoplasias and overexpression of these cofactors may be a contributing factor to the hormone-driven development of neoplasia and to anti-estrogen resistance of breast cancers.

Introduction

Estrogens exert many of their effects through direct binding to a nuclear estrogen receptor (ER). After ligand binding, the ER binds as a dimer to a cognate estrogen response element (ERE) present in the gene promoter regions of estrogen responsive genes. SERMs have tissue specific agonist or antagonist effects leading to the idea that the classical model of ER action is more complex. The discovery of a second ER gene (ER-β), which has a different tissue distribution relative to ER-α, has gone some way to explaining tissue specific effects (Couse et al., 1997; Kuiper et al., 1997). In addition, the recently discovered tissue specific nuclear receptor coactivators and corepressors, which can modify the transcriptional activity of the ER and other nuclear receptors, also play a role in SERM activity (McKenna et al., 1999).

The p160/SRC (steroid receptor coactivator) family is a family of coactivators involved in the regulation of steroid receptor mediated transcription (McKenna et al., 1999). There are three described p160 genes: SRC-1 (Onate et al., 1995), SRC-2 or TIF-2 (GRIP1) (Voegel et al., 1996), and AIB1 (Anzick et al., 1997), also known as (ACTR/RAC3/TRAM-1/SRC-3) (Chen et al., 1997; Li et al., 1997; Suen et al., 1998; Takeshita et al., 1997). Mapping studies have demonstrated that these coactivators have several similar features including a receptor interaction domain (RID), a basic helix-loop-helix/Per/Arnt/Sim homology (bHLH/PAS) domain and a CBP cointegrator interaction site (Glass & Rosenfeld, 2000; Leo & Chen, 2000; McKenna et al., 1999). Of interest for breast cancer was the discovery that an area of chromosome 20q, a region frequently amplified in breast cancer, harbored the nuclear receptor coactivator gene AIB1 (Guan et al., 1996). The AIB1 gene is amplified in 5-10% of breast cancers and the mRNA and protein are overexpressed in approximately 30% of breast tumors (Anzick et al., 1997; Bautista

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et al., 1998; Bouras et al., 2001; Kurebayashi et al., 2000; List et al., 2001a; Murphy et al., 2000). We have determined, using a regulatable AIB1-directed ribozyme, that down-regulation of endogenous AIB1 levels results in loss of estrogen induction of MCF-7 breast cancer cell proliferation *in vivo* and *in vitro* (List et al., 2001a). AIB1 has been shown to directly interact with the ER in breast cancer cells (Tikkanen et al., 2000). Consistent with this, deletion of the AIB1 gene in mice leads to reduced development of the mammary gland (Xu et al., 2000).

An important finding was that a splice variant of AIB1, in which exon 3 is deleted (AIB1-Δ3), is overexpressed in MCF-7 breast cancer cells, as well as in breast tumor tissue (List et al., 2001b; Reiter et al., 2001). The predicted protein product from this isoform is an N-terminally truncated version of AIB1 that has a deleted bHLH domain and most of the PAS region removed (Reiter et al., 2001). Alterations in this domain are of interest since it has been shown that the bHLH/PAS domain can mediate homodimerization between family members and in this case may mediate cross-talk between nuclear receptors and bHLH/PAS family members that have wide ranging effects in cellular growth control (Aravind & Ponting, 1997; Fan & Crews, 1999). Surprisingly, overexpression of the AIB1-Δ3 isoform potentiates ER, progesterone receptor (PR) and epidermal growth factor (EGF) induction of transcription to a much greater extent than AIB1 (Reiter et al., 2001), suggesting that the overexpression of this isoform may well play a role in sensitizing breast cancer cells to hormones during breast cancer development.

In this study we sought to determine how overexpression of AIB1 or the AIB1-Δ3 isoform affected the pharmacology of natural estrogens and SERMs at the ER-α or ER-β. Our data indicate that in breast and endometrial cancer cells and in ovarian cells, hence cells of different

endocrine origins, overexpression of AIB1-Δ3 can cause significant increases in the estrogenic efficacy of natural estrogens and the SERM, tamoxifen, at both ER-α and ER-β. Interestingly, overexpression of either AIB1 or AIB1-Δ3 did not increase the estrogenic efficacy of raloxifene, suggesting that the conformational changes induced by estrogens or partial agonists (Paige et al., 1999) that allow interaction with AIB1-Δ3 do not occur in the presence of raloxifene. Our data, showing the effects on endogenous genes, also imply that overexpression of AIB1-Δ3 could enhance estrogen induced proliferative activity of selected compounds, thus contributing to the development of hormone-dependent tumors and to antiestrogen resistance.

Results

Effects of AIB1-Δ3 on the activation of ER-α

We previously reported that AIB1-Δ3, at saturating levels of 17β-estradiol, can induce a larger estrogen response in a transcriptional assay than full length AIB1 (Reiter et al., 2001). As physiologic hormone interactions often do not take place at saturating hormone levels, we expanded this study and evaluated the response of AIB1-Δ3 to different concentrations of 17β-estradiol, at both ER-α and ER-β. We transfected CHO cells with an expression vector for ER-α, a luciferase reporter plasmid containing a multimerized ERE and with expression vectors encoding either full-length AIB1 or AIB1-Δ3. The transfection of cells with the AIB1 expression vector resulted in a 1.3 fold increase in estrogen activation of luciferase activity, whereas transfection with the AIB1-Δ3 expression vector resulted in a significant 1.9 fold increase in the estrogen response (Figure 1a), thereby confirming the increased efficacy reported by us earlier (Reiter et al., 2001). It is of note that significantly less protein is produced from the transfected AIB1–Δ3 vector than from the full length AIB1 (Figure 1a, Western blot inset), implying that the increase in response on a molar basis is even greater for AIB1-Δ3. Neither AIB1 nor AIB1-Δ3 caused a left shift or a change of the slope of the dose response curve of 17β-estradiol at ER-α and thus only impact on the efficacy and not the potency. The above dose-response curves were generated in the presence of the estrogen antagonist ICI 182,780 at low concentrations to minimize the estrogenic background from serum. We performed similar experiments without ICI 182,780 and observed comparable results, although estrogen induction was lower due to increased background, and the dose response curve was left shifted in the absence of the antagonist. The impact on efficacy by AIB1 and AIB1-Δ3 did not change relative to each other (data not shown). In addition, the amount of DNA transfected is optimal determined by a

titration curve, which showed lesser amounts of DNA were also transfected and coactivation decreased accordingly (data not shown). We therefore chose 1.5 µg of DNA as optimal for all further transfection experiments.

Effects of AIB1-Δ3 on the activation of ER-β

ER-β is widely expressed in normal and malignant tissues (Couse et al., 1997; Kuiper et al., 1997; Roger et al., 2001), but the tissue distribution differs from ER-α (Nilsson & Gustafsson, 2002). Through ER-β, 17β-estradiol can regulate different sets of genes (Couse et al., 1997) and can oppose the effect of ER-α (Hall & McDonnell, 1999). We therefore examined whether AIB1 and AIB1-Δ3 can act as coactivators for ER-β. Using the same experimental design as for ER-α, quantitatively similar results were seen, with a 1.5 fold increase in luciferase activity when AIB1 was cotransfected, and a 2.8 fold increase when AIB1-Δ3 was transfected (Figure 1b). A small, but insignificant left shift of the dose response curve with overexpression of either AIB1 or AIB1-Δ3, indicate a slight increase in potency at ER-β.

Effects of AIB1-Δ3 on genistein mediated activation of ER

Genistein, a phytoestrogen present in soy, produces a wide variety of pharmacological effects and may be responsible for some of the reported effects of soy in the diet. Dietary ingestion of soy has been linked to prevention of breast cancer (Lamartiniere et al., 2002; Zava & Duwe, 1997) and, although the reason for this association remains unclear, it may be due to the estrogenic effects of the genistein in soy. It was therefore of interest to determine the impact of overexpression of the coactivators AIB1 and AIB1-Δ3 on the estrogenic activity of genistein. Transfection of CHO cells with ER-α, an ERE-luciferase reporter and full length AIB1 showed

no significant increase in reporter activity over control-transfected cells in the presence of genistein (Figure 2a). However, when AIB1-Δ3 was cotransfected, genistein activated the reporter 1.6 fold higher than control transfected cells to a maximal 70-fold induction of the promoter, thus enhancing the efficacy of genistein. On the other hand, the potency of genistein at ER-α was not altered by the coactivators. Similar results were observed with ER-β (Figure 2b). In contrast, transfection of AIB1 did not increase reporter activation in the presence of genistein versus control, whereas transfection of AIB1-Δ3 augmented the response 1.7 fold over control (Figure 2b) without effect on the potency of genistein. From this we conclude that AIB1-Δ3 augments estrogenic activity of natural chemopreventive agents such as genistein at relevant serum concentrations in the high nanomolar range (Zava & Duwe, 1997).

Role of AIB1 and AIB1-Δ3 in breast cancer cells

The previous experiments were carried out in CHO cells, which have low background levels of AIB1. To determine if AIB1 or AIB1-Δ3 have an impact on the efficacy of 17β-estradiol in cells with endogenous AIB1, we tested the effects of transfection of these constructs in MCF-7 breast cancer cells. These cells harbor the 20q AIB1 gene amplicon and express both AIB1 and AIB1-Δ3 (Reiter et al., 2001). In these cells, overexpression of AIB1 had no impact on endogenous ER induction of transcription whereas AIB1-Δ3 caused a 2.6-fold increase in 17β-estradiol responsiveness (Figure 3). Thus, the increased efficacy of ER-α with AIB1-Δ3 that we observed in CHO cells is preserved in breast cancer cells.

Role of AIB1-Δ3 in endometrial cancer cell lines

Since expression of cofactors is tissue specific and different subsets of cofactors can alter hormone responses, we investigated the role of AIB1 and AIB1-Δ3 in endometrial cancer cells. Endometrial cancer cell lines show a wide range of expression of AIB1-Δ3, with the lowest levels in Ishikawa cells (about 1% compared to full length AIB1), medium levels in Hec1A cells, and the highest levels in RL95-2 cells (about 10% compared to full length AIB1) (data not shown). Therefore, to minimize the effects of endogenous AIB1-Δ3 we used Ishikawa cells for further experiments. We transfected Ishikawa cells in a similar fashion as described above for CHO cells using a single, saturating dose of 17β-estradiol or genistein for treatment. AIB1 increased the estrogen response with cotransfected ER-α by 1.4 fold, in the presence of 17β-estradiol, whereas AIB1-Δ3 increased the activity 2.2 fold (Figure 4a). AIB1-Δ3 also increased the genistein response by 1.8-fold, while full length AIB1 had no significant effect (Figure 4b). Upon cotransfection with ER-β, in the presence of 17β-estradiol, AIB1 caused a 1.4 fold increase in reporter activity versus a 1.7 fold increase with AIB1-Δ3 (Figure 4c). With genistein, AIB1-Δ3 caused a 2.1-fold increase in activity (Figure 4d), thus demonstrating that AIB1-Δ3 is also a more effective coactivator for estrogens in endometrial cancer cells.

AIB-Δ3 selectively increases the estrogenic activity of 4OH-tamoxifen

Tamoxifen is an effective treatment for estrogen receptor positive breast cancer, however, most responsive tumors will eventually acquire resistance. Furthermore, tamoxifen displays a tissue-selective pharmacology; it is an agonist in some tissues (uterus, bone, liver, and the cardiovascular system) and an antagonist in other tissues (brain and breast) (Jordan, 2002). Both effects, the selective pharmacology and the tumor resistance, could be produced by differential

expression of coregulators, which have been shown to be tissue-specific and functionally distinct. We therefore examined the effect of AIB1-Δ3 on the estrogenic response to 4OH-Tamoxifen (OHT), (the active metabolite of tamoxifen), in CHO, MCF-7, and Ishikawa cells (Figure 5). The cells were transfected with an expression vector for ER-α, a luciferase reporter plasmid containing an ERE and with expression vectors encoding either full-length AIB1 or AIB1-Δ3. When treated with OHT, reporter activity in AIB1 transfected CHO (Figure 5a) and MCF-7 cells (Figure 5c) did not increase compared to control transfected cells. However, when AIB1-Δ3 was transfected, luciferase activity increased 1.5 fold in CHO cells and 2.1 fold in MCF-7 cells treated with OHT (versus control transfected cells) (Figure 5a;c). In OHT treated Ishikawa cells, cotransfection with AIB1 resulted in an increase of reporter activity (1.7 fold over control transfected cells), whereas the transfection of AIB1-Δ3 further augmented luciferase activity 2.3 fold over control (Figure 5e). Raloxifene is another SERM currently approved for treatment of osteoporosis and with potential use in the prevention of breast cancer. In contrast to OHT, overexpression of either AIB1 or AIB1-Δ3 did not significantly increase the estrogenic activity of this compound in any of the cell lines tested (Figure 5b;d;f).

Effects of AIB1-Δ3 on the transcription of endogenous estrogen responsive genes

The previous experiments were all carried with a transfected promoter, which is not in the context of native chromatin. To determine if AIB1-Δ3 also causes increased transcription of endogenous estrogen responsive genes, we examined mRNA levels of Cathepsin D (CATD) and encoding ER-binding fragment-associated antigen 9 (EBAG9) by real time RT-PCR in response to estrogen and tamoxifen in the presence of AIB1 or AIB1-Δ3. Both CATD and EBAG9 harbor classical estrogen responsive elements in their promoters (Augereau et al., 1994; Watanabe et al.,

1998). When AIB1 is transfected into MCF-7 cells estrogen induced transcription of CATD and EBAG9 is only slightly higher compared to the control plasmid (Figure 6). However, transfection of AIB1-Δ3 results in a significant increase of transcription of the target genes, surprisingly. When cells are treated with 4OH-tamoxifen, AIB1-Δ3 also increases the stimulation of transcription of CATD and EBAG9 compared to AIB1 or control plasmid to levels equivalent to17β-estradiol. These results demonstrate that the effects seen in a luciferase reporter assay can be replicated in endogenous estrogen responsive genes, and that overexpression of the AIB1-Δ3 isoform would induce increased estrogenic activity of partial agonists.

Discussion

The data presented in this study confirm and extend our previous findings that, despite its low expression levels, AIB1-Δ3 is a significantly better coactivator of ER-α mediated responses not only in CHO cells, but also in breast and endometrial carcinoma cell lines. This result has implications for breast cancer, suggesting that tumors that express elevated levels of this isoform will be very responsive to endogenous estrogenic stimulation of proliferation and other pro-oncogenic responses mediated through ER-α or ER-β. Interestingly, the estrogenic stimulus is also amplified for a phytoestrogen such as genistein, which is found in soy, which may also lead to increased estrogenic stimulus to the breast in a subset of tumors that contain amplified AIB1 and AIB1-Δ3.

Of significant interest for breast cancer therapy is the observation that the estrogenic activity of the SERM tamoxifen was increased at ER-α in breast cancer and endometrial cancer cell lines. Tamoxifen antagonizes estrogen responses in human mammary epithelium, however resistance to antiestrogen therapy occurs frequently. The mechanism of this increased resistance is not known but a number of investigators have suggested that alteration of cofactors, such as AIB1, required for estrogen receptor action might explain the acquisition of antiestrogen resistance (Osborne et al., 2003). Although full length AIB1 caused no significant increase in tamoxifen's estrogenic activity in any of the cell lines we tested, AIB1-Δ3 caused a significant increase in the estrogenic activity of tamoxifen in both MCF-7 and Ishikawa cells. This suggests that overexpression of this isoform might contribute to the development of antiestrogen resistance in breast cancer cells. The mechanism of the increased transcriptional activity of the AIB1-Δ3 isoform is not known. Experiments comparing the interactions of AIB1 vs AIB1-Δ3 to

components of the transciption complex such as the receptor or CBP have not to date revealed major differences in their binding interactions (unpublished data). We have speculated that another mechanism may explain the increased activity of the AIB1-Δ3 isoform i.e.that loss of the bHLH/PAS dimerization domain leads to loss of interaction with an endogenous repressor (Reiter et al., 2001).

Increased AIB1-Δ3 may also contribute to the increased levels of endometrial carcinoma seen in tamoxifen treated patients (Fisher et al., 1994). Although AIB1 levels in endometrial cancer cells are lower than in breast cancer cells and SRC-1 seems to play a more predominant role in the endometrium (Shang & Brown, 2002), expression of the potent AIB1-Δ3 isoform may increase the estrogenic efficacy of tamoxifen in the endometrium. Indeed it is possible that human endometrial carcinoma contains significant levels of the AIB1-Δ3 isoform and we are currently examining this in human tumors.

Interestingly, neither AIB1 nor the AIB1-Δ3 isoform increased the estrogenic activity of the SERM raloxifene at ER-α, suggesting that neither AIB1 nor AIB1-Δ3 overexpression would play a role in the development of resistance to this SERM. Differences in the ability of the AIB1-Δ3 isoform to coactivate SERMs may explain tumors in which no cross-resistance between these compounds develops. Biochemically, our data suggest that the coactivator binding interface of the ER-α, when bound to tamoxifen, can still significantly interact with the exposed LXXLL motifs of the AIB1-Δ3, but not those of AIB1. In contrast, the binding interface is significantly conformationaly different in the presence of raloxifene, thus preventing interaction with either AIB1 or AIB1-Δ3. Protein crystallography has revealed that estrogen agonists and SERMs

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induce unique conformational states of the ER (Shiau et al., 1998). In the case of estrogen agonists and partial agonists, this may confer preferential binding of certain coactivators or, in the case of antagonists, will prevent coactivator binding and favor corepressor binding (Shiau et al., 1998) More recently it has been reported that differences in the interactions of 4OH-Tam or raloxifene at amino acid 351 of the ER-α cause distinct differences in the estrogen –like action at activating function 2b (Liu et al., 2002). Differences in ER-α confirmation in this domain may mediate changes in AIB1 or AIB1-Δ3 interactions which could differentially enhance estrogen function. It also should be noted that since it is known that raloxifene is an estrogen in bone, this suggests that other factors that play a role in its estrogenic activity are not present in MCF-7, CHO or Ishikawa cells.

It had previously been reported that AIB1 (SRC-3) could significantly coactivate ER-α but had little effect on ER-β (Suen et al., 1998). Therefore, it is somewhat surprising that our data instead show that overexpression of AIB1 has little effect on the efficacy of estrogens or partial agonists at ER-α or ER-β. However, the AIB1-Δ3 isoform increased estrogenic efficacy of 17β-estradiol and genistein at both ER-α and ER-β in all the cell lines studied. ER-β is not expressed highly in malignant breast tissue and its expression appears to go down with progressive malignancy (Roger et al., 2001,4647), therefore its role in the development of antiestrogen resistance may be limited. However, ER-β is expressed at higher levels in bone where raloxifene, and to some extent tamoxifen, cause reversal of osteoporosis. To date, the levels of AIB1 and AIB1-Δ3 in the various cell types of the bone have not been described and it remains to be seen whether these coactivators play a role in estrogenic activity in bone or in other tissues where ER-β is high.

The effect of overexpression of coactivators on the pharmacology of other steroids, such as glucocorticoids, has been extensively examined and in general, an increase in efficacy coupled with a significant decrease in potency is observed, especially for agonists and partial agonists of the glucocorticoid receptor (Szapary et al., 1999). However, in our study of ER-α and ER-β, although small changes in EC_{50} were observed, the general effect of overexpression of the AIB1-Δ3 isoform was an increase in the efficacy of the estrogenic or partial agonist ligands at both ER-α and ER-β. These differences are clearly reflected in changes in endogenous gene expression as seen with the RT-PCR of both EBAG9 and CATD. This suggests that the coactivator complexes and rate-limiting steps of activation are different among nuclear receptors.

Materials and Methods

Reagents

17β-estradiol, 4OH-tamoxifen (OHT) and genistein were purchased from Sigma-Aldrich (St. Louis, MO). Raloxifene was provided by V.C. Jordan (Northwestern University, Chicago). ICI 182,780 was purchased from Tocris (Ballwin, MO). Media, fetal bovine serum (FBS) and Lipofectamine were purchased from Life Technologies, Inc. (Gaithersburg, MD).

Plasmids

The expression vector for human estrogen receptor-α was provided by P. Chambon (CNRS, France). The firefly luciferase reporter plasmid containing the estrogen response element (ERE) from the *Xenopus* vitellogenin gene was provided by V.C. Jordan (Northwestern University, Chicago) and the *Renilla* luciferase vector (pRL-CMV) was from Promega (Madison, WI). The

expression plasmids for AIB1 and AIB1-Δ3 have been described earlier (Reiter et al., 2001). The control plasmid pcDNA3 was purchased from Life Technologies, Inc (Gaithersburg, MD).

Cell culture

All cell lines were obtained from the tissue culture core facility of the Lombardi Cancer Center. MCF-7 cells were cultured in Iscove's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). Ishikawa cells were grown in Mimimum Essential Medium Alpha that was supplemented with 15% FBS. CHO cells were maintained in Ham's F-12 nutrient mixture supplemented with 10% FBS.

Transient Transfection and Reporter Gene Assays

Cells were plated at 25% confluence in twelve-well plates and were cultured for 24 h at 37 °C under 5% CO_2 in the appropriate medium supplemented with 5% FBS that had been treated with dextran-coated charcoal. The medium was then replaced with IMEM containing Lipofectamine Plus and expression and reporter plasmids as indicated. After incubation for 5 h, the medium was replaced with medium containing 5% dextran-coated charcoal-treated FBS and nuclear receptor ligands. Cells were incubated for 24 h and then disrupted in passive lysis buffer (Promega). 20 µl of the resulting cell extract were assayed for both firefly and *Renilla* luciferase activities with the Dual-Luciferase reporter assay system (Promega).

Immunoblot Analysis

Whole cell extracts were prepared as described previously (Harris et al., 2000), and equal portions (30 µg of protein) were resolved on denaturing 4-20% polyacrylamide gradient gels containing Tris-glycine. The separated proteins were transferred to a nitrocellulose membrane and then subjected to immunoblot analysis with a 1:500 dilution of a mouse monoclonal antibody specific for amino acids 376-389 of human AIB1 (Transduction Laboratories, Lexington, KY),

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horseradish peroxidase-conjugated goat antibodies to mouse immunoglobulin (1:10,000 dilution; Amersham Pharmacia Biotech, Piscataway, NJ), and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Real time RT-PCR

Cells were plated at 25% confluence in six-well plates and were cultured in the presence of 10nM ICI 182,780 for 24h at 37°C under 5% CO_2 in IMEM supplemented with 5% FBS that had been treated with dextran-coated charcoal. Cells were then transfected with an expression construct for pcDNA3, AIB1 or AIB1-Δ3 by using Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's instructions. 24h after transfection wells were treated with either ethanol alone, 100nM of 17β-estradiol or 1μM of 4OH- tamoxifen for 2h. The RNeasy Mini Kit (Qiagen) was used for extraction and DNAse treatment of total RNA. Real time RT-PCR was performed using the SuperScript One-Step RT-PCR System (Invitrogen Corp). Samples were reverse transcribed at 50°C for 30 minutes, followed by a denaturing step at 95° for 5 minutes and 40 cycles of 30 sec at 95°C, 30 sec at 55°C and 60 sec at 72°C. Fluorescent data were collected during the 72°C step using the Cycler iQ Detection System (Bio-Rad Laboratories, Inc.). The primers and probes for real-time reverse RT-PCR measurement were: Cathepsin D forward primer: GTACATGATCCCCTGTGAGAAGGT; cathepsin D reverse primer: GGGACAGCTTGTAGCCTTTGC; and cathepsin D probe: 6FAM-ACCCTGCCCGCGATCACACTGA-TAMRA. EBAG9 forward primer: GATGCACCCACCAGTGTAAAGA; EBAG9 reverse primer: AGTCAGGTTCCAGTTGTTCCAAAG; and EBAG9 probe: 6FAM-AGGAGGGAATGGGAATGTGGCAACAC-TAMRA. GAPDH forward primer:

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CCCACATGGCCTCCAAGGAGTA; GAPDH reverse primer: GTCTACATGGCAACTGTGAGGAGG; and GAPDH probe: 6FAM-ACCCCTGGACCACCAGCCCCAGC-TAMRA.

Statistical analysis was carried out using the GraphPad Prism program (GraphPad Software Inc., San Diego, CA).

Acknowledgements

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Figure 1. 17β-Estradiol dose-response curves in CHO cells transfected with coactivator and ER-α or ER-β. **(a)** CHO cells were transfected with either the empty pcDNA3 vector (1.5μg) (Control), pcDNA3-AIB1 (1.5μg), or pcDNA3-AIB1-Δ3 (1.5μg) together with an expression vector for human ER-α (50ng), an ERE-luciferase reporter plasmid (500ng), and pRL-CMV (0.1ng). Cells were incubated for 20 h with the indicated concentrations of 17β-estradiol in the presence of 10nM ICI 182,780 before determining luciferase activity. Relative light units were normalized against Renilla luciferase acitivity. The *inset* shows immunoblot analysis of transfected cell lysates that were fractionated on 4-20% polyacrylamide gradient gels and probed with an antibody to AIB1. **(b)** Cells were transfected, treated and assayed as in **(a)** with the exception that the ER-α vector was replaced with a vector for ER-β (50ng). Data represent mean +/- S.E. of values from three independent experiments, each performed in triplicate. *,+, p<0.01 versus control; #, p<0.01 versus AIB1;

Figure 2. Genistein dose-response curve in CHO cells transfected with coactivator and ER-α or ER-β. **(a)** CHO cells were transfected with either the empty pcDNA3 vector (1.5µg) (Control), pcDNA3-AIB1 (1.5µg), or pcDNA3-AIB1-Δ3 (1.5µg) together with an expression vector for human ER-α (50ng), an ERE-luciferase reporter plasmid (500ng), and pRL-CMV (0.1ng). Cells were incubated for 20h with the indicated concentrations of genistein in the presence of 10nM ICI 182,780 before determining luciferase activity. Relative light units were normalized against Renilla luciferase acitivity. **(b)** Cells were transfected, treated and assayed as in **(a)** with the exception that the ER-α vector was replaced with a vector for ER-β (50ng). Data represent mean +/- S.E. of values from three independent experiments, each performed in triplicate. *, p<0.01 versus control; #, p<0.01 versus AIB1;

Figure 3. Effect of AIB1 and AIB1-Δ3 on transcriptional activity of ER-α in MCF-7 cells. MCF-7 cells were transfected with either the empty pcDNA3 vector (1.5μg) (Control), pcDNA3-AIB1 (1.5μg), or pcDNA3-AIB1-Δ3 (1.5μg) together with an expression vector for human ER-α (50ng), an ERE-luciferase reporter plasmid (500ng), and pRL-CMV (0.1ng). After incubation for 24 h with 10 nM 17β-estradiol in the presence of 10nM ICI 182,780, cells were assayed for luciferase activity. Relative light units were normalized against Renilla luciferase and results are expressed as fold activation over luciferase activity of cells treated with ICI alone. *, $p<0.005$ *versus* the corresponding value for cells transfected with the control vector.

Figure 4. Effect of AIB1 and AIB1-Δ3 on transcriptional activity of ER-α and ER-β in Ishikawa cells. Ishikawa cells were transfected with either the empty pcDNA3 vector (1.5μg) (Control), pcDNA3-AIB1 (1.5μg), or pcDNA3-AIB1-Δ3 (1.5μg) together with an expression vector for human ER-α (50ng), an ERE-luciferase reporter plasmid (500ng), and pRL-CMV (0.1ng). After incubation for 24 h with either 10 nM 17β-estradiol **(a)** or 1 μM genistein **(b)**, both in the presence of 10nM ICI 182,780, cells were assayed for luciferase activity. Relative light units were normalized against Renilla luciferase and results expressed as fold activation over luciferase activity of cells treated with ICI alone. *, $p<0.05$ *versus* the corresponding value for cells transfected with the control vector. **(c)** and **(d)** Ishikawa cells were transfected as in **(a)** with the exception that the ER-α vector was replaced with a vector for ER-β (50ng). After incubation for 24 h with either 10 nM 17β-estradiol **(c)** or 1 μM genistein **(d)**, both in the presence of 10nM ICI 182,780, cells were assayed for luciferase activity. Relative light units were normalized against Renilla luciferase and results are expressed as fold activation over luciferase activity of cells treated with ICI alone. *, $p<0.05$ *versus* the corresponding value for cells transfected with the control vector.
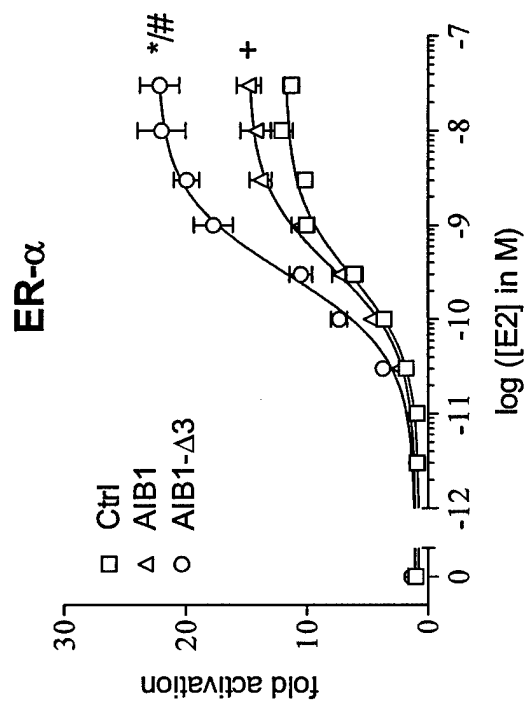
24

Figure 5. Effects of AIB1 and AIB1-Δ3 on the activation of ER-α by OHT or raloxifene in different cell lines. CHO (**a** and **b**), MCF-7 (**c** and **d**) or Ishikawa (**e** and **f**) cells were transfected with either the empty pcDNA3 vector (1.5μg) (Control), pcDNA3-AIB1 (1.5μg), or pcDNA3-AIB1-Δ3 (1.5μg) together with an expression vector for human ER-α (50ng), an ERE-luciferase reporter plasmid (500ng), and pRL-CMV (0.1ng). After incubation for 24 h with 1 μM OHT (**a, c, e**) or 1μM raloxifene (**b, d, f**) in the presence of 10nM ICI 182,780, cells were assayed for luciferase activity. Relative light units were normalized against Renilla luciferase and results are expressed as fold activation over luciferase activity of cells treated with ICI alone. *, $p<0.05$ *versus* the corresponding value for cells transfected with the control vector.

Figure 6. Effects of AIB1 and AIB1-Δ3 on the expression of CATD and EBAG9 in MCF-7 stimulated with estrogen and OHT. Cells were plated at 25% confluence in six-well plates in the presence of 10nM ICI 182,780 in IMEM supplemented with 5% FBS that had been treated with dextran-coated charcoal. After 24 h cells were transfected with either empty pcDNA3 vector (3μg) (Control), pcDNA3-AIB1 (3μg) or pcDNA3-AIB1-Δ3 (3μg). The next day cells were treated with either ethanol alone, 100nM 17β-estradiol or1 μM OHT Tamoxifen for 2 h before RNA was harvested and CATD **(a)** and EBAG 9 **(b)** expression were measured by real time RT-PCR. mRNA levels were normalized by GAPDH mRNA expression, and the normalized values were then expressed relative to that of control cells transfected with pcDNA3 and treated with ethanol alone. Data represent mean +/- S.E. of values from two independent experiments, each performed in duplicate. *, $p < 0.05$ *versus* the corresponding value for control cells.
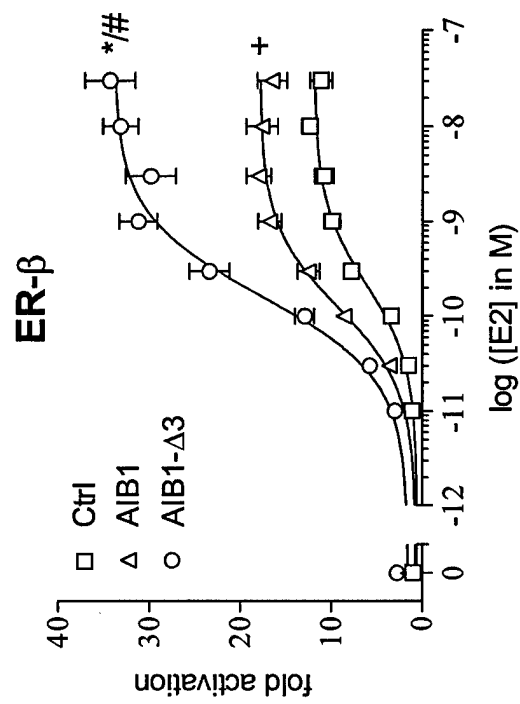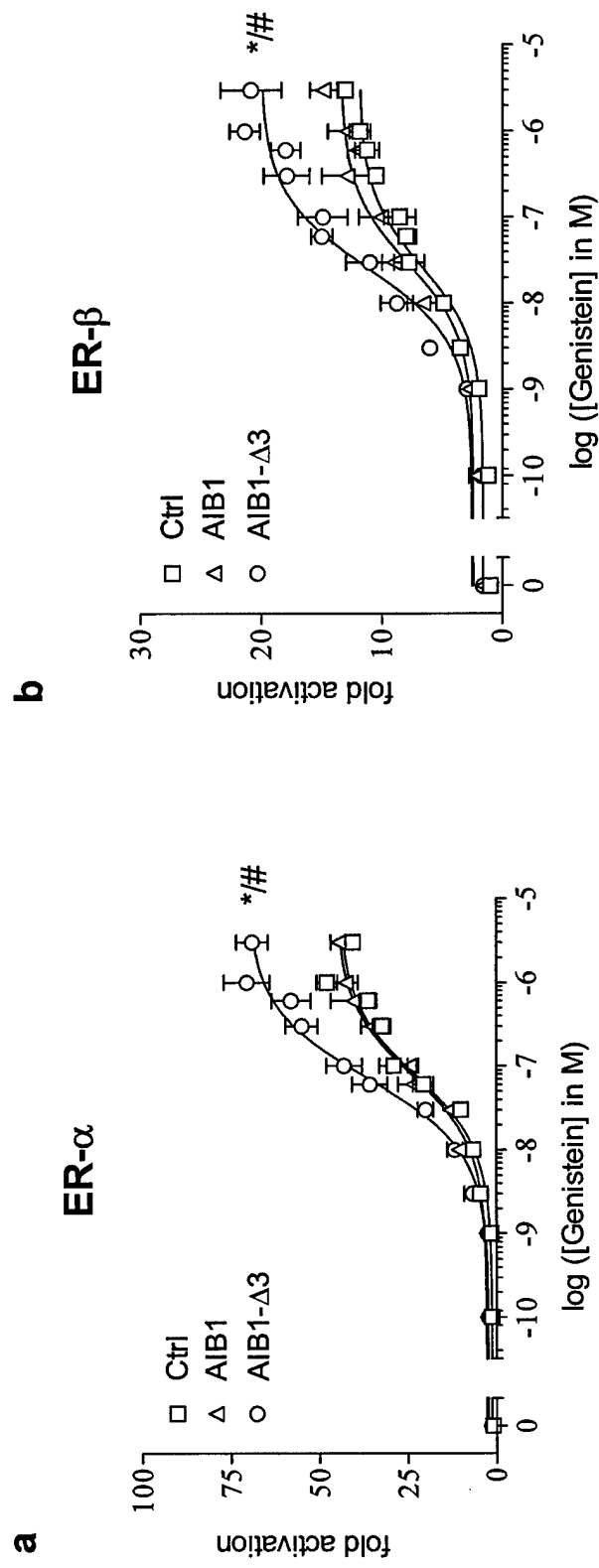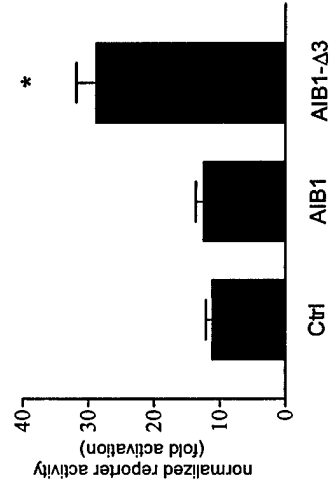
Fig.1.

a

ER-α

□ Ctrl
△ AIB1
○ AIB1-Δ3

fold activation

log ([E2] in M)

b

ER-β

□ Ctrl
△ AIB1
○ AIB1-Δ3

fold activation

log ([E2] in M)

Fig.2.

ER-α

a

fold activation

100
75
50
25
0

Ctrl
AIB1
AIB1-Δ3

0   -10   -9   -8   -7   -6   -5

log ([Genistein] in M)

*/#

ER-β

b

fold activation

30
20
10
0

Ctrl
AIB1
AIB1-Δ3

0   -10   -9   -8   -7   -6   -5

log ([Genistein] in M)

*/#

Fig.3.

MCF-7

Fig.4.

a ER-α, 17β-estradiol

normalized reporter activity (fold activation)

Ctrl   AIB1   AIB1-Δ3 *

b ER-α, Genistein

normalized reporter activity (fold activation)

Ctrl   AIB1   AIB1-Δ3 *

c ER-β, 17β-estradiol

normalized reporter activity (fold activation)

Ctrl   AIB1 *   AIB1-Δ3 *

d ER-β, Genistein

normalized reporter activity (fold activation)

Ctrl   AIB1 *   AIB1-Δ3 *
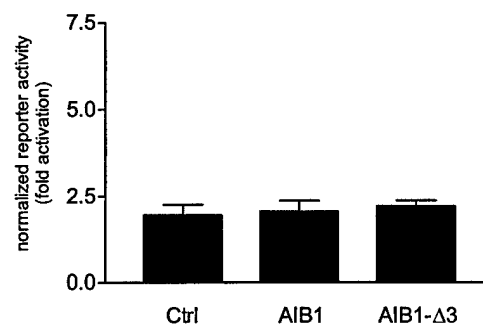
Fig.5.

4OH-Tamoxifen

Raloxifene

CHO

a

b

MCF-7

c

d

Ishikawa

e

f

Fig.6.

a

Cathepsin D

b

EBAG9